Characterization of *Streptomycetes spp* and its Bioactive compounds isolated from Mangrove soil

Final Synopsis

Submitted to
Mangalore University for the fulfillment of the Degree of Doctor of Philosophy
In
Microbiology

Submitted by,

Ms. Akshatha S J

Research Scholar

Reg. No. MU/EXB/Ph.D/CR.37/Micro.Bio/2021-22/E.25

Research Supervisor

Dr. I K Manjula

Assistant Professor

Department of Studies and Research in Microbiology

Jnana Kaveri campus, PG centre Mangalore University

Chikka Aluvara, Kodagu-571232

Contents of the Final Synopsis report

- **General Introduction**
- **Research Objectives**
- **Review of Literature**
- **Chapter-1**
- **Chapter-2**
- **Chapter-3**
- **Chapter-4**
- **Major findings and Conclusion**
- **List of Research Articles published and presented**
- **References**

GENERAL INTRODUCTION

Microorganisms are the diverse group of minute organisms that comprises either a single-celled form or colony of the cells. Microbes have distinct morphological appearances and undergo biochemical transformations to grow in various environments and are extensively dispersed throughout the ecosystem due to their exceptional metabolic capacity aswell as growth accessibility in different ecological conditions (Peterson E and Kaur P, 2018). Microorganisms such as Bacteria, Actinomycetes, Archea, Protists, Fungi, Yeast and Algae are ubiquitously distributed in soil, air and aquatic ecosystem.

Actinomycetes and its significance

The Actinomycetes was derived from greek "atkis (a ray) and mykes (fungus). In the microbial world, Actinomycetes are phylogenetically defined as taxa with high genomic content of guanine and cytosine (55%) subdivision of the gram-positive phylum characterized by the formation of branching filaments in the form of aerial and substrate mycelia with spore chain configuration (Horinouchi S and Beppu T, 2004). They are ubiquitous, free-living saprophytic organisms widely distributed in terrestrial, aquatic, mangrove environments also in association with plants as endophytic organisms which constitute diverse physiological and morphological traits.

Antibiotics: Actinomycetes have been known as the abundant source of antibiotics. Streptomycin was the first discovered aminoglycoside antibiotic isolated from *Streptomyces griseus*. Effective antibiotics such as anthracyclines obtained from *Streptomyces peucetius*, Nocardicins derived from *Nocardia uniformis*, Gentamycin produced by *Micromonospora purpurea* targets the cellular components of pathogenic bacteria and blocks the reproduction phase during replication process.

Enzyme inhibitors: Actinomycetes synthesise enzyme inhibitors of low molecular weight. Leupreptins inhibit papain, plasmin, trypsin and chymotrypsin enzymes produced by *Streptomyces roseoviridis* Revestin inhibits reverese transcriptase enzyme synthesised from *Actinoplanes* and *Streptomycetes* organisms.

Extracellular enzymes: Actinomycetes produces beneficial extracellular enzymes that decompose wide range of organic materials. *Actinoplanes sediminis* produces alkaline protease which breaks down gelatin layers, chitinases produced by *Cryptosporangium*

eucalypti that degrades chitin present in the cellwall of fungal pathogens. *Streptosporangium roseum* produces lipase that hydrolyzes triglyceride molecules and carboxylic ester bond in complex materials.

Streptomycetes **as dominant genera**

Streptomycetes are heterogeneous gram positive, branching, unicellular spore forming filamentous bacteria and represented as one of the largest taxonomic unit under Actinomycetes domain (Chater K F *et al*, 2010). The members of these organisms are placed under group 22 to 29 in Bergey's manual of Determinative Bacteriology. *Streptomycetes* organisms are configured in class Actinobacteria of phylum 14 in Domain II, Bacteria in volume 5. (Clasen J and Bibb M J 2011). Genomic description of *Streptomycetes* displayed high content of guanine and cytosine (>55%) in their genetic material (Shao H *et al*, 2019). Streptomycin was the first antibiotic compound isolated from *Streptomyces griseus* applied for the treatment of tuberculosis infection (Sharma M and Manhas, 2019), Neomycin aminoglycoside antibiotics isolated from soil dwelling *Streptomyces fradiae* inhibits the translation process in gram negative bacteria such as *Escherichia coli*, *Salmonella typhi*, *Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Acinetobacter* (Dow G T *et al*, 2018). Vancomycin is a glycopeptide antibiotic produced by soil-borne *Streptomyces orientalis* affects the methicillin resistant strains of *Staphylococus aureus*, *Staphylococcus epidermidus* and *Mycobacteria* by disrupting their cytoplasmic membranes (Fahmy N M *et al* 2021). In addition to these effective antibiotics *Streptomycetes* species contribute structurally diverse biological compounds such as alkaloids, aliphatic compounds, angucycline, macrolide, thioesters, lactones, biogenic sulphides, mitosanes and peptide molecules having a broad range of biomedical properties for various diseases including atherosclerosis, neurodegenerative diseases, hypocalcemia, chronic respiratory diseases , diabetes, nephrotic syndrome and cancer (Procopio R D *et al*, 2012; Ser L *et al* 2016); Xin Y *et al* 2015).

Occurrence of ***Streptomycetes*** **spp. from Mangrove habitat**

Mangroves are the vegetative system found in coastal region of marine habitat. Due to its unique ecological conditions including temperature, salinity, pH, humidity and soil nutrients, mangroves are the valuable resource to study the microbes that have evolved to specific environmental factors (Singh A R *et al*, 2020). The microorganisms that inhabit in this environment represent potentially rich resource for the isolation and identification of bioactive compounds with pharmacological significance in various therapeutic applications

(Abidin Z A *et al*, 2016). The Mangrove habitat in the Mangalore coast, Dakshina Kannada district, Karnataka, India has rarely been explored for the occurrence of *Streptomycetes* spp. (Karthik Y and M I Kalyani, 2022). The Mangalore coast offers a suitable source for novel strains of *Streptomycetes* with temperature variation ranging from 27°C to 40°C spanning over the year with humid climatic conditions (Dhanya B E *et al*, 2018). Among the members of Actinomycetes, *Streptomycetes* species are recognized to be the dominant genera in mangrove sediments and are strongly influenced by temperature, pH, oxygen level and organic contents (Tan L T *et al*, 2019). These factors may aid in the synthesis of potential primary and secondary metabolites having survival benefits by the microorganisms community. Therefore Mangrove ecosystem provides promising and efficient productive ecosystem in the wet land of tropical latitudes in the coastal area (Law J W *et al*, 2019).

Streptomycetes Antimicrobial peptides as significant Bioactive compound

Streptomycetes species produces commercially important bioactive compounds and have wide attention due to their role in prevention of several chronic infectious diseases (Ramazani *et al*, 2013). Bioactive metabolites required for adaptation are known to be synthesized initially during stationary phase, which constitutes antibiotics, enzymes, peptides and other macromolecules (Benfield A H *et al*, 2020). The synthesis and secretion of these bioactive metabolites either intracellularly or extracellularly are greatly influenced by environmental and genetic factors (Kumar P *et al*, 2018). These metabolites create the building blocks of structural stability during anabolic reactions, also involved in the energy generation process depending on the organisms requirements (Ma H *et al*, 2020).

Among various biological active compound synthesized from *Streptomycetes* species, Antimicrobial peptides (AMPs) are considered as important molecule of interest that must be explored for mechanism based identification of new Antimicrobial peptides (Shai Y *et al*, 2002). Antimicrobial peptides are naturally occurring proteinaceous host defense peptides that are ubiquitously synthesized and present in living forms (Chevrette M G *et al*, 2019). These peptides contain multifunctional therapeutic components that activate innate immune response and function as immune modulators in certain organisms (Sobalosky L *et al*, 2015). AMPs target the bacteria without causing lytic effects to the bacterial cells (Sadreddinamin M *et al*, 2016). It translocates across cytoplasmic membrane without causing damages and inhibits the gene expression at the translation level by binding to specific complementary mRNA sequences leading growth cessation (Lee I *et al*, 2011). It also interferes with

metabolic pathways, induces oxidative stress, blocks the mitochondrial ATP regulated K^+ channel and eventually causes the cell death (Qutb A M *et al*, 2020).

Cancer is the leading cause of death and growing public health threat globally. It is accounted that nearly 1.8 million cancer cases and 9.5 million deaths are reported in the worldwide (Hassanpour S H and Dehghani, 2017). Breast cancer is a tumour that begins from the cells of breast tissues, cells of ducts and lobules (Tanjitjaroenkum J *et al*, 2013). When breast cancer cells penetrate to lymphatic system, there is a greater probability rate, those tumours enters to the blood stream and metastasize to other organs of the body. Majority of the anticancer drugs originated from *Streptomycetes* species are cyclic peptide compounds which allow cancer membrane destabilization, oxidative stress, promotes DNA fragmentation and induces apoptosis (Banerjee A *et al*, 2013). These peptide molecules also exert anticancer activities by various mechanisms including immunogenic cell death, DNA polymerase inhibition, protein unfolding, halting oxidative phosphorylation and anti-angiogenic actions.

On considering this biological interest, the present investigative study on isolation and identification of bioactive compound from *Streptomyces* species isolated using mangrove soil of Mangalore coast. The strains identified in the study showed distinct morphology as analyzed by slide culturing and SEM analysis. The biochemical characteristics of these isolates showed different enzyme activities. This led us to identify five *Streptomyces* species by 16s rRNA gene sequencing analysis. The bioactive peptide compound with respect to antimicrobial activity was extracted from intracellular homogenate following the standard method for protein extraction. In brief, peptides compound were separated upon precipitation using ammonium sulphate salt and salt removal by subjecting to dialysis membrane separation. The purified peptide fractions were separated based on a salt gradient using CM-Cellulose chromatography, RP-HPLC followed by LC-MS spectrometry analysis. The peptide compound of *Streptomyces* species was found to possess effective anti-oxidant activity, anti- anti-inflammatory activity and anti-biofilm activity. Further purified *Streptomyces* peptide compound showed membrane permeabilization effect on *Klebsiella pneumoniae* ATCC 9621 with pore formation and membrane disintegration. This effect on membrane action was confirmed by flow cytometric analysis. The work was further extended to study the cytotoxic potential of *Streptomyces* peptide compound against MCF-7 breast cancer cells which inhibits the cell proliferation and DNA damages by inducing apoptosis.

REVIEW OF LITERATURE

- Actinomycetes constitute a large group of unicellular, filamentous Gram-positive spore-forming bacteria with high proportion of Guanine and Cytosine nucleotide elements (55%) in their genomic DNA. Actinomycetes derived from the Greek words "*Atkis*'' (ray) and "*Mykes*" (fungus). These organisms have physical properties that are transitional between bacteria and fungi. They resemble bacterial characteristics due to the presence of identical cell wall components. Actinomycetes have comparable structural appearances to fungi including spore chain formation with radial vegetative mycelium development. Actinomycetes are widely distributed in various habitats such as soil, aquatic environments, plant litter composed materials and food products. The Taxonomic and ecological positions of Actinomycetes are widely known for their metaboloic flexibility which is frequently accompanied with the synthesis of economically significant secondary metabolites (Isik *et al*, 2014).

- *Streptomyces* is a genus of aerobic filamentous Gram-positive bacteria, enriched with G+C contents in their genome. *Streptomyces* are ubiquitously present in soil, they are distinctly recognized to produce aromatic volatile compound known as "Geosmin" in the moist soil. The morphological differentiation of *Streptomyces* species begins from unicellular, nutrient scavenging mycelium to dormant state of unigenomic spores. When spores encounters the favourable environmental conditions for growth, *Streptomyces* spores germinate by extruding germ tubes and grow into branched hyphal network known as vegetative mycelium. The extracellular matrix of aerial hyphae terminates in the form of elongated spore chains (Hasani A *et al*, 2014).

- (Rattanakavil T *et al*, 2020) reported that hundred Actinomycetes strains isolated from rice field were preliminary screened for antibacterial activity. On the basis of the effective growth inhibition five active *Streptomyces* strains were selected for 16s rRNA gene sequencing analysis for species identification. The BLAST genomic DNA sequences of the isolates were used for phylogenetic tree construction. All the selected Actinomycetes strains belongs to *Streptomyces albidoflavus* NK01101 (99.56%), *Streptomyces hydrogenans* NK08203 (100%), *Streptomyces corchorusli* NK21201 (99.86%), *Streptomyces shenzhenensis* and *Streptomyces jeddahensis*.

- Purification of intra-cellular Bioactive peptide compounds or other metabolites from *Streptomyces* biomass can be generally achieved by the combination of various compound separation techniques such as solid, liquid separation, buffer extraction, solvent extraction, precipitation, absorption, ion-exchange chromatography and size-exclusion chromatography. Intracellular buffer extraction is one of the classical and most versatile compound separation and purification method to recover the biologically active peptide by-products (Pham J V *et al*, 2019).

- Antimicrobial peptides (AMPs) are diverse group amphipathic, hydrophobic cationic small molecular peptides that are essential for the host's innate defence against various bacterial infections. Antimicrobial peptides consist of short polymers of L-amino acids with 15-100 amino acid residues. This peptide typically affect the bacterial membrane by lipid-peptide interactions, by interfering in the intracellular organelles of the bacterial cells, bind to the cytosol, generates free radicals, inhibits electron transport chain reaction by blocking the ATP synthase enzyme, retards the transcription and translation process (Armas F *et al*, 2019).

- (Tornesello A L *et al*, 2020) studied that cancer therapy is current interest in developing novel anticancer agents having different routes of action against cancer cells. Recent studies have shown that cationic Antimicrobial peptides (AMPs) which are toxic to bacterial cells exhibit a broad spectrum of cytotoxicity against cancer cells. The electrostatic attraction between the positive hydrophobic groups of AMPs and negatively charged components of cancer cells are believed to play a major role in strong binding affinity and the selective disruption of cancer cell membranes also regulate the gene expression to induce apoptosis.

- (Tork S E *et al*, 2018) investigated that the marine *Streptomyces pratensis* produces L-glutaminase enzyme having anticancer potential against breast cancer cells. The 3 D model structure of L-glutaminase enzyme composed of two domains, the serine dependent beta lactamase domain and STAS domain (sulphate transporter and anti-sigma factor antagonist). These two domains are linked by a linker peptide. The amino acid sequence alignment possessed 57% protein coverage and belongs to the family glutaminase super-family with highly conserved regions.

CHAPTER- I
Isolation and Morphological Characterization of *Streptomycetes* spp. from Mangrove soil

1. INTRODUCTION

Streptomycetes species are the heterogenous group of Gram positive, soil inhabiting unicellular filamentous bacteria. The genus *Streptomycetes* was proposed by Waksman and Henrici in 1943. These organisms are economically and biotechnologically valuable, as they produces a wide variety of bioactive metabolites including antibiotics, immunosuppressive drugs, antitumor agents and other biologically active compounds (Kripa N V *et al*, 2020).

Mangrove ecosystem have the greatest diversity than the terrestrial habitat, they are characterized by periodic tidal flooding which improves the environmental physiological conditions like salinity, temperature variations, alkaline pH, high pressure, improves the quality of the soil with excess of nutrient transformation from dead mangrove vegetation into sources of carbon, nitrogen, phosphorus, iron, magnesium, calcium and hydrothermal vents (Biswas K *et al*, 2017; Sengupta N *et al*, 2015).

The development of new existing isolation technique, growth conditions and screening methods are required to improve the cultivable isolates. *Streptomycetes* spp. grows slower than bacteria and fungi (Hopwood D A, 2019). Different methods of selective soil pre-treatments such as dilution, chemical treatment and heat treatment methods have been proposed to enhance the growth of mycelium forming *Streptomycetes* spp. by inhibiting unwanted bacterial growth (Shen X *et al*, 2020). Slide culture technique for microscopic observation of spore chain structure gives confirmed information for the identification of *Streptomycetes* organisms, also the International *Streptomyces* project (ISP) suggested that nutrient rich media formulations and physical conditions could be standardized for the cultivation of novel *Streptomycetes* species (Bo A B *et al*, 2019). Considering the above mentioned facts the present study was undertaken to study the isolation and morphological characterization of *Streptomycetes* spp. from mangrove soil.

2. MATERIALS AND METHODS

2.1 Collection of Mangrove soil sediments

The unique habitats of Mangroves in coastal region of Mangalore were recognized. Mangroves vegetation was found in places such as Nethravathi river estuaries, Thokottu and

Thannirbavi river bank estuary around Mangalore coast were selected for collecting the soil samples during the month of January 2019. The collected soil sediments were packed using sterile polythene bags, transported to the molecular research laboratory and subsequently each samples were air dried for 5 days. Stones, shells, plant debris were removed from the dry soil samples. The soil particles were then sieved into coarse powder and stored under room temperature for further analysis.

2.2 Physico-chemical analysis of Mangrove soil samples

The soil samples were examined for physico-chemical parameters. pH of the mangrove soil sediments was determined potentiometrically using pH meter in 1:5 soil-water suspension (potentiometric method). Moisture content of the soil was evaluated by gravimetric method. The soil moisture content was expressed by weight as the ratio of the mass of water present in the dry weight of the soil sample. Available micronutrients such as zinc (Zn), copper (Cu) and iron (Fe) was determined by flame-photometric method. Presence of macronutrients including sodium (Na) and potassium (K) was estimated by flame photometry, phosphorus (P) was estimated by conductometeric method also organic carbon (C) in soil was determined by titration method.

2.3 Growth media for the isolation of mangrove *Streptomycetes spp*

Five different media that supported the growth of *Streptomycetes* isolates were prepared consisting of fluconazole (1mg/ml) and nyastatin (1mg/ml). The media included in the study were starch casein agar media, starch nitrate agar media, yeast malt extract agar (ISP2), glucose asparagine agar media and kenknight munaier agar media. The serially diluted soil samples of 0.1 ml were inoculated at the dilution rate of 10^{-3} and 10^{-5} to each of the above media. The plates were incubated at 30ºC for 7 days and observed periodically for the growth of *Streptomycetes* colonies. Selected *Streptomycetes* isolates were subcultured and standardized to grow on starch casein agar media.

2.4 Enumeration of *Streptomyces* colonies

Total number of *Streptomycetes* isolates obtained by soil dilution plating in all the five media was determined by multiplying the actual number of cells per plate to dilution factor with reciprocal to the volume of soil suspension. Colony count of the *Streptomyces* isolates was expressed in terms of colony forming units (cfu/ml) of the sample.

2. 5 Culture characteristics

Cultural characteristics were studied based on Bergey's manual of determinative bacteriology. After incubation at 30 ºC for seven days mangrove *Streptomyces* isolates grown

on starch casein agar media plates were examined for colony characteristics, shape, elevation, texture, soluble pigment production and colour of the aerial and substrate mycelium.

2.6 Microscopic Examination

(a) Slide culture technique

To study the morphological characteristics of *Streptomycetes* isolates with reference to aerial, substrate mycelium and sporulation, the isolates were cultivated on ISP2 medium by simple inclined coverslip culture technique. An inoculum from a slope culture was then spread along the line and plates were incubated at 37ºC for 4-7 days. The organisms grow on both the medium and along the coverslip. Then coverslip was carefully withdrawn from the medium and observed directly under light microscope at 100X to differentiate between substrate and aerial mycelium.

(b) Gram staining and Acid fast staining technique

As part of the microscopic characterisation of the mangrove *Streptomyces* isolates, gram-staining and acid fast staining techniques were used to analyse Gram positive and non-acid fast organisms.

(c) Field emission- Scanning electron microscopic analysis

Streptomycetes isolates were grown on ISP2 agar medium and cells were fixed in fixative glutaraldehyde for 2-4 h at room temperature, repeatedly washed using phosphate saline buffer and dehydrated the cells using acetone and observed the complete structure under scanning electron microscope at 500X magnification.

2.7 Effect of temperature and pH on growth

Growth parameters of selected Streptomyces isolates were investigated according to the studies recommended by International Streptomyces Project (ISP). *Streptomycetes* isolates were streak inoculated on starch casein agar media slants and placed for incubation at varied growth temperature ranging from 28ºC to 50ºCfor 12 days.. All the tested slants were incubated for 12 days and observed for the growth at different pH and temperature tolerance.

2.8 Antibiotic sensitivity

Streptomycetes isolates were tested for antibiotic sensitivity using amoxicillin (10µg), tetracycline (30µg), polymyxin B (10µg) and amikacin (10µg) that were placed on the lawn cultures and incubated at 30ºC for 7 days. Plates were observed for the sensitive or resistant patterns and recorded.

2.9 Biochemical analysis

Biochemical tests were performed for all the *Streptomycetes* isolates and checked for indole production activity, reduction of nitrate reaction, urease production, MR-VP, sodium chloride tolerance and H_2S production. Also enzyme reactions were checked for starch hydrolysis, catalase activity, casein and gelatin hydrolysis including, citrate utilization and cellulose degradation.

2.10 Extracellular Enzyme production test

The *Streptomycetes* strains isolated from mangrove soil were screened to analyse the secretion of extracellular enzymes such as chitinase, lipase, L-asparaginase, tyrosinase and DNA'se.

2.11 Antibacterial activity by cross-streak method

Cross streaking of the mangrove against *Streptomycetes* isolates human pathogenic strains such as *Bacillus cereus* ATCC 10876, *Proteus vulgaris* ATCC 13315, *Klebsiella pneumoniae* ATCC 9621, *Salmonella typhimurium* ATCC 23564, *Escherichia coli* ATCC 8739 was performed to examine antimicrobial activity. In brief, to the sterile nutrient agar plates all the isolates were line streaked and incubated at 30 ºC for 7 days. After observing the growth, selected bacterial strains were perpendicularly streaked from each corner at equidistant and incubated at 37 ºC for 24 h.

3. RESULTS OF THE STUDY

3.1 Collection of mangrove soil samples and Physico-chemical analysis

Four soil samples collected from mangroves of Mangalore coast were analysed for physicochemical properties of soil colour, texture and water porosity, the collected soil depicted in fig.1. pH values of the soil samples were in the range from 6.2-6.9. Gravimetric soil moisture content was determined by oven drying at 110°C. As shown in Table 1, the percentage of water content was found to be of 7.8-11.36% collected from two different sites at Thokottu from left and right side of the Nethravathy river bridge. Organic carbon was highly distributed in Thokottu (L) mangrove soil. Sodium was found high in Jeppinamogaru, maximum content of phosphorus was found in Tannirbhavi soil and potassium was relatively high in Thokottu (R) mangrove soil. Micronutrient availability of copper zinc and iron was found to be excessive in Thokottu mangrove soil.

3.2 Selective isolation and enumeration of *Streptomycetes spp* from mangrove soil sediments

Soil dilutions from 10^{-3} and 10^{-5} dilutions inoculated aseptically by spread plate technique separately on five growth media such as yeast malt extract agar media (ISP2), starch casein agar media, glucose asparagine agar media, kenknight and munaier's agar media and starch nitrate agar media followed by incubation at 30ºC for 7 days. Periodically observed during seven days of incubation, observed *Streptomycetes* colonies were enumerated using colony forming units (cfu/ml). Total 632 isolates were obtained and selected isolates were maintained for further studies.

3.3 Culture characteristics

From the *Streptomycetes* grown plates, prominent colonies were selected as isolates and sub-cultured for pure culture maintenance. After seven days of growth, the morphological appearances and colony characteristics were studied. Isolates showed abundant growth of branched hyphae, aerial and substrate mycelia on starch casein agar medium. Around 32 percent of *Streptomycetes* mycelium were grey in colour and varied coloration of cream, white, pink and yellow colonies were observed after isolation. Each selected isolates exhibited variation in the colorations, texture of the colony differed from smooth, slimy and dry powdery or leathery appearance, along with or without soluble pigment production.

3.4 Microscopic Examination

(a) Slide culture technique

Streptomycetes spp observation of spore surface morphology by slide culture method showed different arrays of spore chains. The spore bearing hyphal morphology and their arrangements from different isolates revealed the identification of isolates as belonging to *Streptomycetes* genus. Isolates showed bulging branched mycelium arranged with club shaped microconidal spores at the tip of the hyphae. The spores usually of 1-2µm thick varied in terms of shape and size enclosed with sporangial envelope containing numerous matured ovoid and cylindrical spores.

(b) Gram staining and Acid fast staining technique

In Gram staining technique all the selected *Streptomycetes* isolates were determined as Gram positive organisms. The acid fast staining was negative for all the selected isolates and was determined as non-acid fast organisms.

(c) Field-emission Scanning electron microscopic analysis

The SEM images of selected *Streptomycetes* isolates revealed that the internal structure of the sporangium shows clear compact coiled or parallel oriented spores which are bisporous arranged as tubular or cylindrical shaped with long chains of biverticillate circular open spiral filaments. Matured spores of the isolates was varied with grey in colour with length ranges from 20µm in size.

3.5 Optimum temperature and pH

Temperature sensitivity on the growth parameter of the *Streptomycetes* isolates was analysed. All the selected isolates showed efficiency to growth at 28^{o} C and 30^{o} C. Significant number of isolates comprising around 25-35% of the selected isolates can withstand a temperature of 40-45^{o} C.

Similarly, effect of pH is an important key factor to regulate the growth and survival rate of *Streptomyces* species. The *Streptomycetes* strains were allowed to grow in basal media for 7 days at 30ºC. All the selected *Streptomycetes* isolate could withstand pH6.0 and pH 7.0. Based on growth evaluation it was revealed that 40% of *Streptomyces* isolates were able to sustain high alkaline condition and can be considered as facultative alkaliphilc organisms.

3.6 Antibiotic susceptibility test

Antibiotic impregnated discs such as amoxicillin- 10µg, tetracycline -30µg, polymyxin B- 10µg and amikacin-30µg were placed on growth medium that was pre-inoculated with the *Streptomycetes* isolates. The interaction of isolates in the presence of antibiotics to observe sensitivity or resistance was noted. Ten *Streptomycetes* isolates have shown resistant towards all the antibiotics. Thirty *Streptomycetes spp* were found to be sensitive and exhibited clear inhibition zone measured around their growth. It was observed that all the isolates were either sensitive or resistance to all the selected antibiotics.

3.7 Biochemical characteristics

Biochemical tests were carried out for phenotypically identified *Streptomycetes* isolates. Majority of the *Streptomycetes* strains shown positive response to the biochemical analysis such as indole test, starch hydrolysis, catalase test, H_2S production test, nitrate reduction test, urease test, methyl red test, voges proskaeur test, gelatin hydrolysis test, citrate utilization test and cellulose degradation test.

3.8 Extracellular enzyme production

Mangrove *Streptomycetes* isolates were subjected for screening of extracellular enzyme activities for chitinases, lipases, L-asparaginases, tyrosinases and DNA'ses by spot inoculation on suitable agar medium. Nearly 50% of the isolates were responded for the production of extracellular enzyme activities

3.9 Preliminary screening of antibacterial activity by cross-streak method

Thirty nine Mangrove *Streptomycetes* isolates except MY 29 showed antibacterial activity against *Bacillus cereus* ATCC 10876, *Proteus vulgaris* ATCC 13315, *Klebsiella pneumonia* ATCC 9621, *Salmonella typhimurium* ATCC 23564, and *Escherichia coli* ATCC 8739.

4. DISCUSSION

The mangroves of the Mangalore coastal region are among one of the most unexplored sites for the *Streptomycetes* genus. *Streptomycetes* is the largest group under the Actinobacteria phylum of Actinomycetes. The Mangalore coastal regions are exploited with rare *Streptomycetes* species exhibited with various metabolic activity and produces diverse bioactive compounds. To record on the physicochemical nature, each of the soil samples was subjected to analysis such as texture, pH, moisture and macro/micronutrients compositions. Textures of the soil differed from sandy, silt, loamy and clay soil. The soil sample collected from Thokottu region was clay and silt in texture with pH of 6.9. Also sandy texture was observed from Tannirbhavi soil sample with pH of 6.7. Near the Nethravathi river estuary, the mangrove soil was found as loamy soil with pH 6.8. Available micronutrients such as zinc, iron and copper were estimated by flame photometry and conductometric method. potassium, sodium, phosphorus and organic carbon macronutrients were analyzed in each soil samples. Similarly in previous study non- rhizospheric soil samples were subjected for pH analysis, organic carbon and nitrogen availability, further studied for the isolation and enumeration of Actinobacteria using starch nitrate media (Veleso J M *et al*, 2017),.

Streptomyces species characterization was achieved upon spore pattern, isolates grown on cover-slip by slide culture technique and staining with lactophenol cotton was microscopically examined that resulted in the selection of forty prominent *Streptomyces* genus. The isolates were checked for the positive or negative reactions for biochemical tests in comparison with referring to the Bergey's manual of determinative bacteriology, the isolates were confirmed as belonging to *Streptomyces* species. Correspondingly in previous

studies indigenous *Streptomyces spp* have shown positive results for catalase test, nitrate reduction test, triple sugar iron agar test following Bergey's manual of determinative bacteriology for provisional identification. All the *Streptomycetes* isolates except MY29 significantly inhibited the growth of test pathogens namely *Klebsiella pneumoniae* ATCC 9621, *Salmonella typhimurium* ATCC 23564, *E.coli* ATCC 8739, *Staphylococcus aureus* ATCC 23565, *Bacillus cereus* ATCC 10876 and *Proteus vulgaris* ATCC 13315. (Li F *et al*, 2019) has evaluated the antibacterial efficacies of *Streptomycetes* isolates by cross streak techniques as primary screening and effectively supressed the growth of pathogens namely *E.coli, Staphylococcus aureus, Salmonella typhi* and *Enterobacter* sp. Based on the above literature evidence, our present investigation confirms that the potential *Streptomycetes* strains isolated from Mangrove soil are morphologically diverse and significantly affect the pathogenic test organisms.

CHAPTER- II

Intra-cellular peptide extraction from mangrove *Streptomycetes* isolates and screening for antimicrobial and related bio-activity

1. INTRODUCTION

Microbial infections and pathogenesis have developed resistance against currently available antibiotics in recent years (Chemoh W *et al*, 2021). Pathogens counteract the inhibitory property of antibiotics and various antimicrobial compounds through various routes of mechanism by producing virulent enzymes that degrade and inactivate the target drug and efflux of antibiotics resulting in lower intracellular accumulation (Gullon and Mellado, 2018). Numerous antibiotic profiles have found ineffective against multi-drug resistant bacteria and thus has created an imbalance between the control of infections due to multi-drug resistant pathogens and available antimicrobial compounds (Kotrbova L *et al*, 2022).

Drug resistant bacteria such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Acinetobacter baumanii*, *Enterococcus faecalis* and *Mycobacterium tuberculosis* have acquired alternate pathways to hydrolyze the β-lactam antibiotics namely penicillin G, quinolones, vancomycin and levofloxacin (Nguyen *et al*, 2019). These mechanistic abilities of infecting microbes to overcome the effectiveness of the antibiotics are the major clinical issue. In view of the above existing problems, it is necessary to explore effective antibacterial

compounds of natural origin (Chung B *et al*, 2020). *Streptomycetes* derived peptide compound have shown significant importance as antimicrobial peptides against multi-drug resistant bacteria (Bhakyashree K and Kannabiran K, 2018). *Streptomycetes* spp. are reported to produce various antimicrobial peptide compounds including oleandomycin isolated from *Streptomyces antibioticus*, Josamycin obtained *Streptomyces naronensis*, erythromycin *Streptomyces erythreus*, 6-dimethyltetracycline procured from *Streptomyces aureofaciens*. Prolonged consumption of steroid drugs results in adverse physiological side effects like inflammation, oxidative stress and increase in the risk factors of cancer etc (Saleem M *et al*, 2009). To overcome these problems, the Antimicrobial peptides isolated from *Streptomycetes* organisms are found to play a lead role in manifesting the biological functions including anti-oxidant, anti-cancer, anti-inflammation and anti-biofilm properties by neutralizing the side-effects (Ferraiuolo S B *et al*, 2021). Therefore in the present chapter we have studied on peptides from reliable source such as *Streptomycetes* sp. from unique environment as that of mangrove soil may prove useful in identifying antimicrobial peptide compound. Significant members of the *Streptomycetes* genus are screened for producing antibacterial compound and other biological active compounds that have been clinically implicated.

2. MATERIALS AND METHODS

2.1 Culturing of *Streptomycetes* isolates for Biomass Production

Streptomycetes spp were mass cultivated in yeast malt extract broth (ISP2) prepared using malt extract 8.0g/l yeast extract 5.0g/l dextrose 2.0g/l. Media was introduced with supplements of nyastatin (1µg/ml) and fluconazole (1µg/ml). All the flasks were incubated in rotary shaker at 28ºC and 120 rpm speed for 14 days.

2.2 Intracellular Bioactive peptide compound extraction from mangrove *Streptomycetes* isolates

After fermentation process biomass was separated from centrifugation at 8000 rpm for 15 min at 4°C. Mycelium biomass was homogenized using 0.1M sodium phosphate buffer pH 7.2. A clear supernatant was obtained after centrifugation for 15 mins. Ammonium sulphate (18 g) was gradually added to 50 ml clear supernatant solution and kept overnight at 4ºC for complete precipitation. The protein precipitates were collected by centrifugation at 8000 rpm for 30 min in 25 mM sodium phosphate buffer pH 7.2. The protein precipitates were desalted using dialysis membrane against 25 mM sodium phosphate buffer pH 7.2.

2.3 Antibacterial activity by well diffusion method

Antibacterial activity of intracellular peptide metabolites obtained from *Streptomyces* isolates was performed by agar well diffusion method The test organisms such as *Bacillus cereus* ATCC 10876, *Proteus vulgaris* ATCC 13315, *Klebsiella pneumoniae* ATCC 9621, *Salmonella typhimurium* ATCC 23564, *Escherichia coli* ATCC 8739 were inoculated on media plates, wells were made using 6mm cork borer about 50µl of each intracellular bioactive metabolites of *Streptomyces* isolates were loaded into the wells aseptically. Antibiotic chloramphenicol (1µg/ml) was used as positive control and allowed for incubation at 37 ºC for 24 h.

2.4 NATIVE PAGE analysis

Native-PAGE was carried out using 10% polyacrylamide gel. After Electrophoretic run at 150V for 2 h, gel was carefully removed and peptides were stained by 0.1% coomassie brilliant blue R 250 in a mixture of 10% acetic acid and 25% methanol for 1 h. Gel was washed several times with sterile water and destained using 10% glacial acetic acid and methanol solution to visualize the bands.

2.5 Cationic amino acid detection from intracellular *Streptomyces* peptide extract by TLC

To detect the presence of cationic aminoacids in peptide fractions thin layer chromatography was performed. Peptide extract and standard amino acids was spotted on the line of silica coated chromatography sheets. Solvent system was used as mobile phase. The TLC set up was allowed for the partition separation of aminoacids. Chromatography sheets were sprayed using ninhydrin reagent and dried at 110°C for the complete evaporation of solvent. Separated aminoacids were observed visually and compared with standard amino acids by measuring retention factors (R_f) of active spots.

2.6. Antioxidant activity

2.6.1 2, 2-Diphenyl-1 picrylhydrazyl Radical scavenging activity (DPPH)

Free Radical scavenging capacity of the peptides from *Streptomyces* isolates was determined using 2, 2-Diphenyl-1 picrylhydrazyl Radical scavenging (DPPH) assay. 2 ml methanolic DPPH solution was added to peptide extracts (20µg-100µg/ml). The reaction mixture was incubated for 30 mins in the dark. The absorbance of scavenging activity of antioxidants on DPPH radicals was measured at 520 nm. The Scavenging activity of the DPPH radical was calculated using the formula

$$\text{DPPH Radical Scavenging activity } \% = [(A_0-A_1)]/A_0] \text{ X } 100$$

A_0- Absorbance of the control, A_1-Reaction system of the test sample and ascorbic acid.

2.6.2 Lipid Peroxidation assay

Thiobarbituric acid reactive species (TBARS) assay was employed to estimate the inhibitory potential of peptides compound from *Streptomyces spp* against lipid peroxidation using egg yolk homogenate as lipid rich media. Malondialdehyde tetrabutylammonium salt (1mg/ml) was taken as standard. The percentage inhibition of lipid peroxidation was measured at the absorbance 520 nm and calculated from the formula. Inhibition (%) = $[(A_{control}-A_{sample})/A_{control}]$ X 100.

2.7 In vitro Anti-Inflammatory activity

2.7.1 Inhibition of albumin denaturation

Invitro anti-inflammatory activity of peptides from *Streptomyces* isolates was evaluated by albumin denaturation method. Aspirin drug (1mg/ml) was used as standard. Protein denaturation inhibition absorbance was measured at 660 nm. Percentage inhibition of denaturation was calculated by the formula

% Inhibition of protein denaturation = $[(A_{control}-A_{sample})/A_{control}]$ X 100

2.7.2 Determination of membrane stabilization activity

Membrane stabilizing activity was performed using goat erythrocyte. RBC suspension was treated with peptide extracts of *Streptomyces* isolates. The reaction mixture was incubated at 37°C for 30 mins and centrifuged at 3000 rpm for 10 mins. The test supernatant solution was estimated at 560 nm. Diclofenac was taken as standard drug. Percentage of erythrocyte membrane stabilization was calculated using the formula

% Inhibition= $[(A_{control}-A_{sample})/A_{control}]$ X 100

2.8 Biofilm inhibition assay

2.8.1 Detection of Biofilm producing bacteria by congo red agar method

Congo red agar is the selective media to identify the biofilm producing organisms. Sterile congo red agar plates were inoculated with *Klebsiella pneumoniae* ATCC 9621, *Staphylococcus aureus* ATCC 23565 and *Bacillus cereus* ATCC 10876. All the plates were incubated for 24 h at 37°C. After incubation colour of the colonies were observed.

2.8.2 Microscopic visualization of biofilm

Overnight cultures of biofilm positive bacterial strains treated with peptide extracts of *Streptomyces* isolates were allowed to grow on cover slip placed in 24 well polystyrene plates. After 24 h of incubation coverslips were carefully removed and stained with crystal

violet. Stained coverslips were placed on glass slides and examined for biofilm formation under phase contrast microscope at 100 X magnification.

2.8.3 Determination of biofilm inhibition concentration

24 h old bacterial suspension were seeded into 96 well microtitre plates and incubated for 4 h at 37° C to promote cell attachment. The planktonic bacterial cells were discharged by washing twice with PBS and treated with peptide extracts of *Streptomyces spp* followed by 24 h of incubation. Bacterial cells of which biofilms formed on the walls of the wells were stained with crystal violet and plates were maintained at room temperature for 15 mins. Then crystal violet stained wells were washed using PBS and allowed for drying at 60°C for 1 h. After drying process wells were solubilized with 30% glacial acetic acid. Chloramphenicol (1µg/ml) was taken as reference standard. Percentage of biofilm inhibition was measured at the absorbance 590 nm and calculated using the formula

$$\% \text{ Inhibition} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

3. RESULTS OF THE STUDY

3.1 Mass culture and intracellular bioactive peptide extraction from mangrove *Streptomyces spp.*

On the basis of potential antibacterial activity by cross streak method twenty selective *Streptomyces* isolates were cultured in production media ISP2 for bioactivity determination. After a maximum growth on the 14th day of the fermentation the produced biomass was separated. The biomass was separated from the media broth and homogenized for intracellular homogenate extraction using 0.1 M phosphate buffer. Intracellular homogenate of the isolates was subjected to salting-in using ammonium sulphate up to 60% saturation. This step resulted in the precipitation of protein components present in the homogenate. These precipitated fractions were salted out by using a dialysis membrane against phosphate buffer and the fractions were resolubilized in a buffer as dialysate extract. An aliquot of the dialysate extract was estimated for the presence of protein by Lowry's method.

3.2 Antibacterial activity by well diffusion method

Among twenty *Streptomyces* sps, eleven intracellular peptide extracts of *Streptomyces* isolates showed antibacterial activity against *Bacillus cereus* ATCC 10876, *Proteus vulgaris* ATCC 13315, *Klebsiella pneumonia* ATCC 9621, *Salmonella typhimurium* ATCC 23564, and *Escherichia coli* ATCC 8739.

3.3 Thin Layer chromatography for the detection of cationic aminoacids from peptide extracts of *Streptomyces* isolates

Thin layer chromatography is reliable and qualitative method to identify the cationic aminoacids from *Streptomyces* peptide extracts. After spraying ninhydrin reagent on TLC sheets separated peptides appeared as blue spots having specific positive signals for aminoacids and compared the coinciding R_f values of *Streptomyces* peptide fractions.

3.4 Antioxidant activity

3.4.1 2, 2-Diphenyl-1 picrylhydrazyl Radical scavenging activity (DPPH

The percentage inhibition of free radical scavening activity of peptides extracts obtained from *Streptomyces* isolates was evaluated by DPPH Radical scavenging activity. The absorbance of the *Streptomyces* peptide reaction mixture showed a great variation with increase in the percentage of inhibition and exhibits significant free radical scavenging activity compared to Ascorbic acid (standard).

3.4.2 Lipid peroxidation inhibition assay

Lipid radicals formation is due to the oxidative destruction reaction by free radicals on polyunsaturated fatty acids results in the damage of biological membrane that leads to membrane disfunction. In order to distract this propogation reaction peptides of *Strepotmyces* isolates were used as antioxidants to inhibit the lipid peroxidation. In the present study we assesed to quantify the level of malondialdehyde (MDA, lipid peroxidation marker) in the lipid rich egg homogenate. Peptide fractions of *Streptomyces* isolates showed maximum lipid peroxidation inhibition with significant reduction of MDA level in egg homogenate.

3.5 Determination of anti-inflammatory studies by *Streptomyces* AMPs

3.5. 1 Inhibition of protein denaturation

Protein denaturation is the major cause of inflammatory and arthritic diseases. The present findings investigated the ability of *Streptomyces spp* peptides to inhibit albumin denaturation. The peptide compound was tested at various concentration and observation revealed that as the concentration increases percentage of albumin denaturation was found to increase and compared to standard drug inhibition.

3.5.2 Invitro membrane stabilization activity

RBC membrane stabilization effect by *Streptomyces* peptide extracts reduces the inflammatory responses by preventing hypotonicity induced haemolysis. The peptide extracts of *Streptomyces* inhibits the lysis of erythrocyte membrane in goat blood sample and results were compared with the standard drug sodium dichlofenac.

3.6 Inhibition of bacterial cell adhesion from peptide extracts of *Streptomyces* isolates

3.6.1 Selection of Biofilm forming bacteria

On a congo red agar plate bacteria such as *Klebsiella pneumoniae* ATCC 9621, *Staphylococcus aureus* ATCC 23565 and *Bacillus cereus* ATCC 10876 displayed black colour colonies with dry crystalline appearance indicating that are biofilm makers.

3.6.2 Biofilm examination under Phase contrast microscopy

Streptomyces spp peptide compound were employed to study the suppression of test bacterial biofilms on coverslip glass surfaces. Bacterial cells with out the treatment of peptide extracts were observed as normal cell morphology with smooth surfaces. Under the same growth conditions in the presence of peptide extracts from both the *Streptomyces spp* bacterial cells were visualized as disaggregation in colonization on the surfaces and found small bacterial clusters under phase contrast microscope at 100 X magnification.

3.6.3 Inhibition of Biofilm formation using microtitre plate method

Antibiofilm effectiveness of peptides from *Streptomyces spp* were tested against biofilm forming bacteria *Klebsiella pneumoniae* ATCC 9621, *Staphylococcus aureus* ATCC 23565 and *Bacillus cereus* ATCC 10876. Results of the assay showed that peptide extracts of *Streptomyces spp* decreased the bacterial species ability to form biofilms.

4. DISCUSSION

In recent years, antibiotic resistant bacteria are rapidly emerging and considered as the major problem in controlling pathogenic infections. The resistant strains of microbes eventually developed by various biological modulations including vascular membrane potential to antimicrobial agents and bioactive compound degradation by producing effective enzymes. Due to this, several life-threatening infections have occurred and the available lead compounds are becoming inefficient as antimicrobial activity.

In the present study, we investigated the occurances of *Streptomycetes* organisms in mangrove soil of Mangalore coastal region. *Streptomycetes* isolates were standardized to grow on starch casein agar media for pure culture maintenance. On starch casein agar medium, *Streptomycetes* isolates exhibited distinctive morphological features with efficient mycelial growth. Antimicrobial peptide extraction from twenty *Streptomycetes* isolates were achieved upon mass cultivation of *Streptomycetes* isolates using production media yeast malt extract broth (ISP2) pH 7.2. After fourteen days of maximum cultivation, intracellular

components from the cultured biomass was harvested by centrifugation at 8000 rpm for 10 mins and homogenized with 0.1M sodium phosphate buffer. To isolate intracellular peptide from homogenate fraction, ammonium sulphate salt precipitation was performed at 60% saturation. After complete precipitation, residual salts were removed by dialysis technique. This resulted in the partial purification of the intracellular peptides from *Streptomycetes* isolates. Further the protein concentration of the antimicrobial peptide compound was estimated by Lowry's method using bovine serum albumin (BSA) as a standard. Similarly, in earlier studies (Peng F *et al*, 2020), reported that *Streptomycetes* strains were isolated from rhizosphere soil of *Panax notoginseng*. *Streptomycetes* isolates were mass cultivated using glucose yeast malt extract broth (GYM) pH 7.2. and extracted the antimicrobial peptide compound. The resulting intracellular peptide compound was tested for antibacterial activity by well diffusion method, among twenty *Streptomycetes* isolates, eleven intracellular peptide extracts from *Streptomycetes* isolates such as MY1, MY5, MY12, MY13, MY14, MY16, MY23, MY26, MY27, MY33 and MY36 showed significant antibacterial efficacy against all the test pathogens. In previous investigation, (Hamed M *et al*, 2021) reported that the bioactive compounds obtained *Streptomyces mutabilis* have shown wide spectrum of antibacterial activity against *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella typhimurium* 14028 and *E.coli* ATCC 19404 with growth inhibition ranged from 12.0±0.9 to 20±1.9mm.

Biological activities of the peptide compound from MY1 and MY16 *Streptomycetes* isolates showed the significant effect on antioxidant, anti-inflammatory and anti-biofilm activity. The observations revealed that as the concentration of the peptide compound increases the percentage of inhibition was also found to increase. In this study maximum 80% of inhibition was observed at 100µg/ml concentration. Similar obsevation was found in the earlier report, the bioactive compound extracted from *Streptomyces* MUM292 has biological potential in inhibiting free radicals, inflammatory constituents and bacterial adherence at maximum concentration 80µg/ml (Tan *et al*, 2019).

The Antimicrobial peptide compound of MY1 and MY16 *Streptomycetes* isolates revealed the broad spectrum of antibacterial activity against all the test pathogens also these organisms are regarded as the potent resource for the identification of new lead peptide compounds that exhibit substantial level of anti-oxidant, anti-inflammatory and anti-biofilm properties.

CHAPTER-III

Molecular Identification of Mangrove *Streptomycetes* spp.

1. INTRODUCTION

Streptomycetes are the important bacterial genus for the bioactive compound production. During the last decades, isolation of new *Streptomycetes* strains and production of novel biological components from the members of this genus are finding extremely difficult due to low yield of genomic DNA, high polysaccharides contaminants, incomplete cell lysis, and poor efficiency of DNA extraction techniques (Hamid M E, 2021). Taxonomic characterization of *Streptomycetes* species is more complicated and challenging compared to the other microbial genera (Handayani N S, 2021). The techniques for the identification and characterization methods have evolved from classical morphological screening to molecular aswell as phylogenetic characterization with gene sequencing analysis which targets linear 16s rRNA gene sequences due to the advancement of polymerase chain reaction (PCR), DNA-DNA hybridization (DDH) and whole genome shotgun sequencing methods (Badadi Z K *et al*, 2022).

International *Streptomyces* project (ISP) is one of the standard scientific approaches for the classification of *Streptomycetes* organisms. ISP was amended and enacted by the committee on taxonomy of the American society of Microbiology and International committee on bacteriological nomenclature in 1964 (Kim Y P *et al*, 2003). Phylogenetic tree are generated by neighbour joining (NJ) algorithm which is classified into two types such as distance-based and character-based methods. Character based technique optimizes the distribution of actual data of individual gene sequence (Chaves J V *et al*, 2017). Considering the above mentioned information, in the present chapter we have identified the potential *Streptomycetes* strains in molecular level by 16s rRNA gene sequencing analysis.

2. MATERIALS AND METHODS

2.1 Molecular identification of mangrove *Streptomycetes* isolates by 16s rRNA gene sequencing analysis

DNA of *Streptomyces* isolates was extracted by CTAB (Cetyltrimethyl ammonium bromide buffer) method using microbial DNA isolation kit (Bogar Bio bee stores Pvt Ltd). Precipitated DNA was homogenized using TE buffer. PCR reaction was carried out to amplify the 16s rRNA genes from genomic DNA and the amplicons were sequenced in

Applied Biosystem sequence scanner software. The amplified PCR product was analysed by 1% agarose gel electrophoresis. Nucleotide sequences of 16s rRNA gene from *Streptomyces* isolates were submitted for Basic local alignment search tool algorithm (BLAST)) and the resultant sequence were aligned using CLUSTAL W 3.7 program version. Gene sequences of the *Streptomyces* isolates were deposited to GENBANK accession number.

3. RESULTS OF THE STUDY

3.1 16s rRNA gene sequencing

16s rRNA gene sequences was deposited to NCBI GENBANK nucleotide data base. Amplified 16s rRNA genes were aligned using CLUSTAL W software and phylogenetic tree was constructed by neighbour joining method based on the NCBI BLAST database available at http://www.ncbi.nim.nih.gov. Sequence similarities of isolates were compared to evolutionary distance of reference *Streptomyces* species in genomic data base bank and deposited under GENBANK to procure accession number for identified *Streptomycetes* isolates as *Streptomyces minutiscleroticus*, *Streptomyces spinoverrucosus*, *Streptomyces albogriseolus*, *Streptomyces flavotricini* and *Streptomyces geysiriensis*.

4. DISCUSSION

The *Streptomycetes* organisms isolated from mangrove soil in Mangalore coastal region have been characterized based on the morphological appearances, biochemical, extracellular enzyme secretion and phylogenetic features.

The potential five *Streptomycetes* strains such as MY1, MY5, MY16, MY23 and MY26 were selected for molecular identification by 16s rRNA gene sequencing analysis. The genomic DNA from five *Streptomycetes* isolates were extracted by CTAB method and 16s rRNA gene was amplified with appropriate PCR conditions using gene specific forward 27F and reverse 1492R primers. DNA was assessed and 16s rRNA gene was imported to Basic local alignment search tool algorithm (BLAST) accessible through National centre of Biotechnology information (NCBI) database (http://www.ncbi.nim.nih.gov). MY1 *Streptomycetes* isolate was identified as *Streptomyces minutiscleroticus* with 92% identity under the accession number MW479445, MY5 isolate showed 45% *Streptomyces spinoverrucosus* under the accession number MZ621832, MY16 isolate revealed 100% relatedness to *Streptomyces albogriseolus* under the accession number MW479446, MY23 isolate exhibited 49% similarity to *Streptomyces geysiriensis* under the accession number MZ621831 and

MY26 isolate found 58% similarity to *Streptomyces flavotricini* under the accession number MZ621830. In several reports phylogenetic analysis of *Streptomycetes* species gave a vast phenotypic description to study the species differentiation. (Maria B F *et al*, 2018) investigated that the molecular identification of 16s rRNA gene sequence of the isolate AA1 had shown 100% similarity to *Streptomyces lincolensis* detected in phylogentic analysis and their nucleotide sequence was deposited in the GENBANK database using BLAST tool. In another previous report, the active strain SN-3 was identified as *Streptomyces gancidicus* with 92% sequence similarity and 16S rRNA gene sequence was deposited to genomic data base bank and procured the accession number NBRC154 (Nayaka S *et al*, 2018). The five prospective *Streptomycetes* strains were therefore identified by polyphasic approach in the current investigation and they represent interesting possibilities for the discovery of advanced bioactive peptide compounds for diagnostic applications.

CHAPTER-IV

Reverse Phase-HPLC, LC-MS, N-terminal Amino acid sequencing analysis of *Streptomycetes* Antimicrobial peptides and determination for Anti-cancer activity

1. INTRODUCTION

Antimicrobial peptides (AMPs) are natural small molecular host defense peptides having multifunctional therapeutic molecules known to be essential components for the innate immune response to participate in certain organisms as immune modulators (Gyawali R *et al*, 2014). With indiscriminate abuse of antibiotics, pathogens-acquired resistance has emerged one of the world's greatest health threats. Emergence of resistance is attributed due to inactivation of antimicrobials caused by bacterial enzymes, lower permeability of bacterial cell membrane and drug inactivation (Santos J C *et al*, 2018). To overcome these obstacles natural AMPs considered as promising candidates because of their remarkable mode of action which is different from that of synthetic antibiotics (Powers J P *et al*, 2003). Mangrove origin *Streptomycetes* genus is a gram positive bacteria remain as unexploited and luxuriant source for active peptide compounds and secrete various intracellular antibacterial molecules with multiple bioactivities and potent clinical efficacy (Ding T *et al*, 2019). *Streptomycetes* organisms are reported to produce various Antimicrobial peptide compounds namely lenticulone produced from *Streptomyces* sp JP 95, mediomicin B isolated from *Streptomyces*

mediocidicus, lajollamycin extracted from *Streptomyces nodosus*, chromomycin B produced from *Streptomyces coclicolor* and metacycloprodigiosin isolated from *Streptomyces longispororuber* DSM 40599 execute different mode of bactericidal action against multi drug resistant pathogens (Maiti P K *et al*, 2020). Despite significant advancements in cancer therapy, cancer cells gaining resistance to the existing conventional anticancer medications (Hoskin and Ramamoorthy 2008). As a result cellular alterations such as increased expression of drug-detoxifying enzymes, drug transporters and increased ability to repair the DNA damage also defects the cellular machinery that mediates apoptosis (Moustafa G O *et al*, 2021). Because of the development of resistance by cancer cells, numerous studies have demonstrated that natural cationic Antimicrobial peptide compounds which are toxic to bacterial cells but not to mammalian cells exhibit a broad spectrum of cytotoxicity against cancer cells (Kim I W *et al*, 2021).

Based on the above mentioned facts, the present chapter was studied with the identification and characterization of Antimicrobial peptide compound isolated from *Streptomyces minutiscleroticus* and *Streptomyces albogriseolus*. The active peptide compound was examined for bacterial membrane permeabilization effect and tested anticancer potential against MCF-7 cancer cells.

2. MATERIALS AND METHODS

2.1 Purification of *Streptomycetes* Antimicrobial peptide compound by CM-Cellulose Ion- Exchange chromatography

Peptide purification was performed by ion exchange chromatography to separate the molecule on the basis of net surface charge. The Dialysed peptide sample was loaded on to column of carboxy methyl (CM) cellulose. Adsorbed peptides were step wise eluted in a series of 0.1M, 0.5M, 1M and 1.5M linear NaCl concentration gradient at the flow rate of 1.0ml/min. The eluted peptides were monitored using *Thermofischer multisky scanner* at 280 nm. Based on highest absorbance the peptide elutions were pooled as different peak fractions.

2.2 Molecular weight determination

Non denaturing NATIVE PAGE was carried out in a 10% polyacrylamide gel according to. The electrophoretic run was performed at 4˚C. Peptides stained using 0.1% coomassie brilliant blue R-250 (w/v) in a mixture of 10% acetic acid and 25% methanol for 30mins. Once the staining is complete, the gels were destained using 10% acetic acid solution and determined the peptides molecular weight.

2.3 Antibacterial activity for *Streptomyces* peptide peak fractions

Agar well diffusion method was performed to check antibacterial activity of the CM-cellulose pooled fractions. The eluted pooled peaks of 50µl were loaded to nutrient agar wells. After incubation at 37ºC for 24 h the antibacterial activity was examined for the zone of inhibition around the wells. A standard control chloramphenicol (1µg/µl) was compared.

2.4 Reverse-phase HPLC separation of peptide compound

Peptides fractions collected by CM-cellulose Ion exchange chromatography were injected to C18 Reverse-phase column for the absolute separation and purification of peptides and fractions were collected upto 20 mins of retention time.

2.5 Identification and characterization of *Streptomyces* peptide compound by Liquid Chromatography –Mass spectrometry (UPLC/ESI-QTOF-MS)

HPLC peptide fractions of *Streptomyces* isolates were subjected for Liquid chromatography coupled to mass spectrometry. Mass detection positive polarity electrospray combined ionization source was used to analyse desired peptide mass range.

2.6 Effect of *Streptomyces* peptide compound on bacterial cell membrane

Klebsiella pneumoniae ATCC 9621 were used as model organisms to study the cell membrane damage. Peptide compound of *Streptomyces* isolates were treated to bacterial culture and fixed with 2% glutaraldehyde. After fixation and drying process bacterial morphological changes was observed under Field emission scanning electron microscope.

2.7 Quantification of cell membrane damage by *Streptomyces* peptide compound

Klebsiella pneumoniae ATCC 9621 cells were grown in 96 well plates in nutrient broth medium and incubated for overnight at 37ºC. Bacterial cells were treated with *Streptomyces* peptide fractions and incubated for 12 h. After incubation the percentage of PI uptake by DNA positive cells was determined and data analysis was studied from the histograms by flow cytometry. Ciprofloxacin was used as standard reference drug.

2.8 Detection of Apoptosis in *Klebsiella pneumoniae* ATCC 9621 by acridine orange staining.

Peptide fracions of *Streptomyces* isolates treated to *Klebsiella pneumoniae* ATCC 9621 was analysed by dual staining assay and visualized under fluorescence microscope to detect the bacterial cells undergoing apoptosis when compared to untreated normal bacterial cells. Treatment with standard drug ciprofloxacin was assayed for cell death.

2.9 Cytotoxic activity of *Streptomyces* peptide compound

The human breast (MCF-7) cancer cell line was obtained from National centre for cell science (NCCS), Pune India. Cancer cells were cultured in Dulbecco's modified eagle

medium (DMEM) supplemented with fetal bovine serum (10%) and penstrip (1%) solution. The cells were incubated at 37°C with 5% CO_2 in humidified atmosphere until cancer cell confluence reaches to 80-90%.

2.10 Detection of apoptotic cells by TUNEL assay

Apoptotic cancer cell DNA degradation induced by peptide fractions of *Streptomyces* isolates was performed using TUNEL assay. Human breast cancer cell line (MCF-7) was maintained by growing on 6-well plates. Further cells were treated with the IC_{50} concentration dose of *Streptomyces* peptide extract and allowed for incubation at 37°C with 5% CO_2 in humidified atmosphere. Cancer cell DNA was labelled by incubating the cells with TUNEL reaction mixture and the percentage of TUNEL-positive cells was determined from the histograms by flow cytometry.

2.11 Amino acid sequence prediction by Maldi-MS analysis

Antimicrobial peptide compound of *Streptomyces albogriseolus* (pk5) was subjected for MALDI-MS (ABI SCiex 5800 TOF/TOF system) to analyse the N-terminal aminoacid sequencing. The purified peptide compound were dissolved in equal proportion of matrix composed of trans 3, 5 dimethoxy 4 hydroxy cinnamic acid, 30% acetonitrile and 0.1% trifluroacetic acid (1:1:1 v/v). 100µl of the sample mixture was deposited to MALDI-target plate in the positive refractor mode. The amino acid sequence data was processed with protein database using the software MASCOT distiller (2.1, Matrix science).

2.12 Identification of *Streptomyces albogriseolus* pk5 peptide compound structure

N-terminal amino acid sequence of *Streptomyces albogriseolus* (pk5) peptide compound generated from MALDI-MS analysis was submitted in SWISS-Prot database to construct the peptide compound structure. The software searches the database for similarity with the query structure. Based on the sequence similarity the best suited Antimicrobial peptide compound structure was updated.

3. RESULTS OF THE STUDY

3.1 Peptide separation by ion exchange chromatography

The solubilized peptides of *Streptomyces* isolates were separated by ion exchange chromatography using CM-cellulose column. Adsorbed peptide was step wise eluted in 0.1-2.0M NaCl salt concentration gradient. Peptide fractions were eluted with the significant peaks at 280 nm by 0.5 M NaCl elution was pooled as peak fractions. Prominent peaks of were found at 280 nm were pooled as peak fractions.

3.2 Molecular weight determination of *Streptomyces sps* peptide fractions by NATIVE - PAGE

Purified peptide fractions of *Streptomyces* isolates were estimated for the molecular weight by NATIVE-PAGE separation. The peptides were separated on 10 % resolving gel and found distinct bands of molecular weights when compared with the standard molecular weight marker.

3.3 Antibacterial activity for peptide pooled peak fractions

CM-Cellulose eluted peptides in the peak fractions were checked for antibacterial activity by well diffusion in comparison with chloramphenicol. The prominent peptide peak fractions of *Streptomyces* isolates revealed significant antibacterial activity.

3.4 Reverse phase HPLC separation of *Streptomyces sps* peptide fractions

Active peptide fractions were subjected for RP-HPLC for the separation of large hydrophobic peptide molecules. Purity of the peptide compound was verified and analysed by HPLC chromatogram. pure active peptide fractions of *Streptomyces* isolates emerged with two dominant peaks were collected at the flow rate of 0.5ml/min.

3.5 Liquid Chromatography- Mass Spectrometry

Purified peptides were subjected to LC-MS revealed the presence of peptides showing a single predominant peak of monomeric subunits with a mass charge ratio of *Streptomyces minutiscleroticus* peptide pk5 of 10000 m/z 31.27 kDa, *Streptomyces albogriseolus* pk4 about 6000 m/z 24.87 kDa and pk5 about 5580 m/z 28.61 kDa were detected.

3.6 Bacterial cell-membrane damage by FE-SEM analysis by *Streptomyces* peptide compound

Klebsiella pneumoniae ATCC 9621 were used as model organisms to study the cell membrane damage. Overnight culture of log phase bacterial cells was treated with *Streptomyces* peptide fractions. Treated bacteria undergone cell wall disruption, shrinkage in the cytoplasm with membrane disintegration. Chloramphenicol was used as standard drug.

3.7 Flow cytometric analysis of Bacterial cell membrane damage

Peptide fractions of *Streptomyces albogriseolus* pk5 showed the remarkable cell membrane damage effect compare to other isolates in FE-SEM analysis and further we have choosen these fractions to quantify the PI uptake cells. *Klebsiella pneumoniae* ATCC 9621 cells were grown in 96 well plates in nutrient broth medium and incubated for overnight at 37ºC. Bacterial cells were treated with *Streptomyces albogriseolus* pk 5 peptide fractions and

incubated for 12 h. The percentage of PI uptake by DNA positive cells was determined and data analysis was studied from the histograms by flow cytometry analysis.

3.8 Fluorescence imaging for the detection of cell death

Klebsiella pneumoniae ATCC 9621 cell suspensions ($0.5X10^6$ cfu/ml) was treated with *Streptomyces albogriseolus* pk5 peptide fractions and kept for 24 hours of incubation. Dual staining was carried out by 1:1 mixture of acridine orange (1mg/ml) and ethidium bromide (1mg/ml) and examined the dead cells.

3.9 Cytotoxicity of *Streptomyces* peptide compound on cancer cells

Peptide fractions of *Streptomyces albogriseolus* pk5 showed prominent cytotoxic effect against MCF-7 cell lines. Variation in cellular morphology of MCF-7 cells were visualized under inverted phase contrast microscope after the treatment of *Streptomyces* peptide extracts. Cisplatin was used as standard reference medication. The results of inhibitory impact of pk5 peptide fractions from *Streptomyces albogriseolus* showed significant toxicity against MCF-7 cells.

3.10 Quantification of TUNEL positive MCF-7 cells

Peptide fractions pk5 from *Streptomyces albogriseolus* were chosen to confirm DNA fragmentation by TUNEL assay on the basis of strong toxicity results by MTT reduction experiment against MCF-7 cancer cell lines. After 24 h test compound treatment significantly exhibited chromosomal DNA breakages and it was compared to standard drug cisplatin in MCF-7 cells.

3.11 Aminoacid sequencing analysis of *Streptomyces albogriseolus* pk5 peptide compound

N-terminal aminoacid sequencing of *Streptomyces albogriseolus* pk5 peptide compound obtained after Maldi-MS analysis were assembled using Mascot Distiller software (2.3, 2017 version, Matrix Science). The total assembled aminoacid sequencing had a length of 202 aminoacid residues. Aminoacid query was searched using pBLAST against Swiss Prot database, the target sequence of the peptide compound showed 68% sequence similarity to *Cryptococcus neoformans* and thus the taxonomy of the *Streptomyces albogriseolus* pk5 peptide compound belongs to the family "Imidazoleglycerol-phosphate dehydratase" (Fig.55). The Antimicrobial peptide compound belongs to the family "Imidazoleglycerol-phosphate dehydratase" is the first report identified from *Streptomyces albogriseolus* having effective antibacterial efficacy and cytotoxicity against MCF-7 cancer cells.

3.12 Structure elucidation of *Streptomyces albogriseolus* pk5 peptide compound by SWISS-Prot database

N-terminal amino acid sequence of the *Streptomyces albogriseolus* pk5 peptide compound was submitted to SWISS-Prot database. The software searches the database for similarity with the query structure. Based on the sequence the peptide compound structure was constructed. The homology modelling of the peptide compound structure demonstrates that typical octa-topology of *Streptomyces albogriseolus* pk5 peptide compound were folded into α and β helical structure comprising 20 antiparallel α helices and parallel 15 β sheets.

4. DISCUSSION

In recent years, antibiotic resistance bacteria are rapidly emerging and considered one of the major problem in controlling microbial infections. The resistant strains of microbes eventually developed different biological modulation such as membrane permeability to antimicrobial agents and compound degradation by enzymes. Due to this, various life threatening infections have occurred and the available lead compounds are becoming ineffecient as antimicrobial activity. *Streptomycetes* derived antimicrobial peptides have broad spectrum of antibacterial activity against multi-drug resistant bacteria. Various reports have demonstrated that catioinc antimicrobial peptides are toxic to bacteria exerts a wide range of cytotoxic effect against human cancer cells.

Antimicrobial peptide purification was carried out by ion-exchange chromatography on carboxy methyl (CM)-cellulose with 0.1 M sodium phosphate buffer pH 7.2. Bound proteins which showed antibacterial activity was eluted by a series of 1 and 2M NaCl gradients were pooled and the NATIVE-PAGE protein profile showed the presence of a single major peptide band with low molecular weight in the peak fractions Previous studies was performed similarly for isolating serratiopeptidase from *Streptomyces hydrogenans*. Intracellular proteins were solubilized using sodium borate hydrochloric acid buffer pH 9.0 followed by dialysis and further purification was carried out by loading on to sephadex G-100 column (Nageshwara S *et al*, 2019).

Purified mangrove *Streptomyces* peptide peak fractions were identified by LC-MS analysis. The mass charge range showed the single predominant peak at various retention times. The peak fractions obtained from *Streptomyces albogriseolus* pk4 showed 24.87 kDa, pk5 showed 28.61 kDa and *Streptomyces minutiscleroticus* pk5 showed 31.27 kDa. This

peptide peak fractions showed significant antimicrobial activity was further assessed to analyse the bacterial cell permeabilization. *Klebsiella pneumoniae* was used as model organisms. Morphological and cell wall disruption was observed in FE-SEM image. Similarly in previous work formation of pores, ambiguous cell boundaries and cytoplasmic breakage of peptide treated bacteria was reported (Wang *et al*, 2018). Bacterial membrane distintegration was quantified by flow cytometry analysis. Peptide compound coated with TUNNEL reaction mixture and PI penetrate inside the bacterial cells tend to accumulate mainly in the nucleus of cells by causing significant membranolytic effect of 21.3% PI cell uptake comparing with Ciprofloxacin (1mg/ml) 23.5 % PI cell uptake.

In earlier studies *Staphylococcus aureus* cells treated with antimicrobial peptides of *Rana aravilis* showed 40% membrane permeability with significant uptake of PI followed by intracellular metabolites leakage through permeabilized membranes and dead cells were encountered after 60 mins of exposure (Dhaneesha M *et al*, 2020).

Cytotoxic activity from purified peptide fractions of *Streptomyces minutiscleroticus* pk5, *Streptomyces albogriseolus* p4 and pk5 revealed the significant toxicity against MCF-7 breast cancer cell lines. Morphological cell damage and increased in apoptosis was found high at the IC50 dosage 72.59µg/ml from fractions *Streptomyces albogriseolus* pk5 fractions. In previous report anticancer compound from marine *Streptomyces akiyoshiensis* GRG 6 showed the potential cytotoxicity against MCF-7 at the concentration 250 µg/ml (Roncevic *et al*, 2019).

Thus the occurances of *Streptomyces* species in the mangroves of Mangalore coastal region offered as one of the natural resource for identification of novel bioactive peptide compound having potential biological property with cell membrane permeabilizing ability and their leading to cell death process in the bacterial cell model of *Klebsiella pneumoniae* ATCC 9621.The structure of *Streptomyces albogriseolus* pk5 Antimicrobial peptide compound structure of was obtained by N-terminal amino acid sequence using SWISS Prot database and the findings revealed that the isolated compound belonged to the family ‘Imidazoleglycerol-phosphate dehydratase”. Similarly in the earlier investigation, the purified chitinase enzyme from *Streptomyces albidoflavus* were analyzed for N-terminal amino acid sequence, the gene encodes a protein of 453 amino acid residues to other chitinases in the protein database and the enzyme showed 70% similarity with chitinase from *Streptomyces plicatus* and belongs to “glycoside hydrolase family”(Das P *et al*, 2017).

MAJOR FINDINGS AND CONCLUSION

In the present study, Mangrove soil samples were collected from the Mangalore coastal region at different location that included Nethravathi river estuaries, Thokottu and Thannirbavi river bank estuary. The soil sediments were pre-treated with 1% calcium carbonate ($CaCO_3$) solution. Soil samples were analyzed for physico-chemical properties including soil colour, texture, moisture content and estimated the available micronutrients and macronutrients. A total of 632 *Streptomycetes* colonies were obtained and quantified by colony forming units (CFU/ml). Among these, 40 morphologically divergent colonies were selected and sub-cultured using five specialized media. Among the selected media starch casein agar showed the abundant aerial-substrate mycelial growth and sporulation of the isolates.The *Streptomycetes* diversity was evaluated based on phenotypic features, enzyme hydrolysis, biochemical characterization and antibiotic sensitivity analysis. Antibacterial activity by cross streak method was performed to select the potential *Streptomycetes* strains, which were then subjected to intracellular buffer homogenization and carried out dialysis for Antimicrobial peptide (AMP) compound extraction. The isolated peptide compound was tested for antibacterial activity by well diffusion method. Out of twenty *Streptomycetes* strains, eleven isolates showed significant growth inhibition against *Klebsiella pneumoniae* ATCC 9621, *Salmonella typhimurium* ATCC 23564, *E.coli* ATCC 8739, *Bacillus cereus* ATCC 10876 and *Proteus vulgaris* ATCC 13315.

Among eleven *Streptomycetes* isolates MY1, MY13, MY16, MY26, MY27 and MY33 isolates inhibited the test organisms at 10µg concentration and no visible bacterial growth was found on nutrient agar plates. The Antimicrobial peptide extracts of MY1 and MY16 *Streptomycetes* isolates were examined for anti-oxidant, anti-inflammatory and anti-biofilm activities which showed potential inhibition of free radicals, reduced protein denaturation, stabilize membrane lysis and hydrolysed the adhered bacterial group. Based on growth stability, morphological appearances and antibacterial efficacy, Five potential *Streptomycetes* strains were selected for 16s rRNA gene sequencing analysis. The intracellular Antimicrobial peptides of *Streptomyces minutiscleroticus* and *Streptomyces albogriseolus* was subjected to ion exchange chromatography separation using CM-cellulose column. The active bound peptides which showed antibacterial activity eluted by a series of 1.5M and 2M NaCl gradients were pooled as peak fractions. The peptide fraction was estimated by Lowry's method using BSA as standard. The Antimicrobial peptide compound of *Streptomyces minutiscleroticus* pk5, *Streptomyces albogriseolus* pk4 and pk5 were

examined for membrane damaging effect on *Klebsiella pneumoniae* ATCC 9621 cells. The *Streptomyces minutiscleroticus* pk5, *Streptomyces albogriseolus* pk4 and pk5 were also examined for cytotoxic activity against MCF-7 cancer cells by MTT assay. All three peptide compounds significantly inhibit the cancer cells proliferation. Based on the maximum inhibitory concentration *Streptomyces albogriseolus* pk5 peptide compound were selected to analyse the cancer cell DNA damaging effect that induces 11.8 % of DNA breakages at 72.59µg/ml concentration. Further our work was extended to identify the structure of bioactive peptide compound of *Streptomyces albogriseolus* pk5. N-terminal aminoacid sequencing analysis possessed 202 aminoacid residues with 68% sequence similarity to *Cryptococcus neoformans* which belongs to the family ‘‘Imidazoleglycerol-phosphate dehydratase.

The current study highlighted that the peptides from mangrove *Streptomycetes* species obtained through intracellular extraction can be considered as effective bioactive peptide compound. The intracellular *Streptomycetes* bioactive peptides are attributable to antibacterial, anti-oxidant, anti-inflammatory and anti-biofilm activities. Also, with respective to the cytotoxic activity, bioactive peptide compound of *Streptomycetes* species showed significant toxicity against MCF-7 breast cancer cells mediated by inducing apoptosis. Thus the peptides obtained are considered as antimicrobial peptide also exerts anticancer activity in MCF-7 cells. The *Streptomycetes* species obtained from mangrove soil of Mangalore coastal region could serve as promising resource with immense antimicrobial peptides biosynthetic potential. Thus the future research and development in the field of *Streptomycetes* derived antimicrobial peptides could be the effective natural drug against multi-drug resistant bacteria and cancer diseases.

Publications of Research Articles

1. Akshatha S J, Manjula Ishwara Kalyani*, Yamanappa Hosmani. Isolation and extraction of antimicrobial peptides from *Streptomyces minutiscleroticus* and *Streptomyces albogriseolus* from Mangrove soil of Mangalore coast, Karnataka. *Ind. J.Nat. Prod. Res*. 2022, 13(1), 1-12. IF-0.54 (Scopus)
2. Akshatha S J, Manjula Ishwara Kalyani*. Mangrove-soil *Streptomyces sps* exhibiting culture and biochemical variation for determining antibacterial activity. *J.appl.Microbiol*, 2022. 16 (4), 1-19. IF-1.05 (Scopus)
3. Akshatha S J, Manjula Ishwara Kalyani*. Microbial Antimicrobial peptides as Anti-cancer agents with special reference to *Streptomyces* species. Futuristic trends in Biotechnology, Iterative Internationl publishers. 2022 (Book Chapter)
4. Akshatha S J, Manjula Ishwara Kalyani*. Bacterial Biofilm inhibition and anticancer activities of cationic peptides isolated from Mangrove *Streptomyces albogriseolus*. *International Journal of Biological Macromolecules*. 2022. IF- 6.0 (Scopus) (Research paper is under communication)

Research papers presented in International and National conferences

1. Akshatha S J, Manjula Ishwara Kalyani*. Molecular Identification and antibacterial effect of *Streptomyces albogriseolus* MW479446 from Mangrove soil. National conference on Advances in Microbial Biotechnology: Current trends and future prospects held at Jnana Bharathi campus Bangalore University, Bangalore. Dated from 22-04-2022 to 24-04-2022. **(Oral Presentation)**
2. Akshatha S J, Manjula Ishwara Kalyani*. Studies on diversity of Bioactive metabolites producing Actinomycetes isolated from mangrove sediments, Dakshina Kannada district. International conference on Recent trends and Advances in Biomedical sciences-An integrated approach. 42nd Annual International conference of Indian Association of Biomedical scientists (IABMS) held at Nitte KS Hegde Medical college, Mangalore. Dated from 12-11-2021 to 15-11-2021. **(Oral Presentation)**

Participation in Seminars/ Conferences and Workshops

1. Participated in seven days hands on training program on **Pre-clinical drug screening** sponsored by DST-STUTI held at Dr. Bhanuben Nanavathi pharmacy college, Mumbai-2022.Dated from 6-7-2022 to 13-7-2022.

2. Attended one day National Webinar on **Integrated Microbiological and Biotechnological Approaches for Scientific Advancements (IMBSA).** Dated 12-04 -2022.

3. Participated in three days of online workshop on **Advancement in Applied Microbiology and Biotechnology (WAMB)**. Dated from 9-02-2021 to11-02-2021.

4. Attended International webinar on **Current status of vaccine development and Immunization**. Dated 3-12-2020.

5. Attended two days **International workshop on Biology and Applications of Actinomycetes** held at Manasa Gangothri campus, University of Mysore, Mysore. Dated from 16-12-2019 to 17-12-2019.

REFERENCES

- Abidin ZA, Malek N A, Zainuddin Z and Chowdhury A J, Selective isolation and antagonistic activity of actinomycetes from mangrove forest of pahang, Malaysia, *Front Life Sci* 2016, 9(1): 24-31
- Armas F, Décor L M, Hamparez K C. Lipopeptide extraction from *Streptomycetes* and their Biological activites . *PLOS ONE* 2018, 2: 1-17
- Benfield A H, Henriques S T. Mode of action of Antimicrobial peptides: membrane disruption vs intracellular mechanisms. *Front. Med. Technol* 2020, 2: 1-10
- Chater K F, Biro S, Lee K J, Palmer T, Schrempf H. The complex extracellular biology of *Streptomyces*. *FEMS Microbiol Rev* 2010, 34 (2): 4-12
- Chevrette M G, Carlson C M, Ortega H E, *et al*. The Antimicrobial potential of *Streptomyces* from insect microbiomes. Nat Commun, 2019 (10) 1-11
- Das P, Kumar P, Kumar M *et al*. Purification and molecular characterization of chitinases from soil actinomycetes. *African journal of Microbiology research* 2017, 11 (27): 1086-1102
- Dow G T, Thoden J B, Holden H M. Three –dimensional structure of NeoB: An aminotransferase involved in the biosynthesis of neomycin. *Protein Sci* 2018, 27(5): 945-956
- Dhaneesa M, Naman BC, Krishnan KP, *et al. Streptomyces artemisiae* MCCB 248 isolated from Arctic fjord sediments has unique PKS and NRPS biosynthetic genes and produces potential new anticancer natural products. *3 Biotech* 2017, 7(32): 1-10
- Fahmy N M, Tawab A M, Hary S, *et al*. Isolation and characterization of marinesponge–associated *Streptomyces* sp. NMF6 strain producing secondary metabolite(s) possessing antimicrobial, antioxidant, anticancer, and antiviral activities. *Journal of Genetic Engineering and Biotechnology* 2021,9: 1-14
- Hamed M, Abdrabo M A, Fahmy NM, *et al*. Distribution and Characterization of Actinomycetes in Mangrovehabitats (Red sea, Egypt) with special emphasis on *Streptomyces mutabilis* M3MT483919. *J Pure Appl Microbiol* 2021, 15(1): 246-261
- Hasani A, Kariminik A, Khosrow I. *Streptomycetes*: Characteristics and their Antimicrobial activities. *International Journal of Advanced Biological and Biomedical research* 2014, 2(1): 63-75
- Hoskin D, Ramamoorthy A. Studies on anticancer activities of antimicrobial peptides. *Biochimica et Biophysica Acta* 2008, 2: 357-375

- Karthik Y, Kalyani M I. Occurrence of *Streptomyces tauricus* in mangrove soil of Mangalore region in Dakshina Kannada as a source for antimicrobial peptide. *Journal of basic Microbiology* 2022, 62: 1-15
- Ma H, Hu K, Su P. Antimicrobial peptide of *Candida albicans* and quantification of cell membrane lysis. *Antibiotics* 2020, 3: 2509-2520
- Maria B F, Sharmili A, Anbumalarmathi J, Isolation and characterization of actinomycetes from marine soil. *MOJ Biol Med* 2018, 3(6), 221-225
- Moustafa G O, Shalay A, Naglah A M, *et al*. Synthesis, Characterization, In vitro Anticancer potentiality, and antimicrobial activities of novel peptide–glycyrrhetinic-acid-based derivatives. *Molecules* 2021, 26:457-469
- Nageswara S, Guntuku G, Yakkali B L. Purification, Characterization and Structural elucidation of serralysin like alkaline metalloprotease from a novel source. *Journal of Genetic Engineering and Biotechnology* 2019; 17 (1): 2-15
- Nayaka S, Dandin C J, Babu G K, *et al*. Molecular Characterization and Antimicrobial efficacy of *Streptomyces gancidicus* strain SN-3 from soil samples. *Journal of Microbiology Research* 2018, 8(4): 97-102
- Peng F, Zhang M Y, Hou S Y, Chen J, *et al*. Insights into *Streptomyces* spp. isolated from the rhizospheric soil of *Panax notoginseng*: isolation, antimicrobial activity and biosynthetic potential for polyketides and non-ribosomal peptides. *BMC Microbiology* 2020, 20 (143): 1-16
- Procopio R D, Silva I R, Martins M K, *et al*. Antibiotics produced by *Streptomyces*. *Braz J Infect Dis* 2012, 16(5): 466-471
- Ramazani , Moradi S, Sorouri R, *et al*. Screening of Antibacterial activity of *Streptomyces* species isolated from Zanjan province, Iran. *International journal of Pharmaceutical, Chemical and Biological sciences* 2013, 3(2): 342-349
- Santos J C, Sousa R C, Otoni C G, Moraes A R, *et al*. Nisin and other Antimicrobial peptides: production, mechanisms of action, and application in active food packaging. *Innovative Food science and Emerging technologies* 2018, 48:179-194
- Ser H L, Tan L T, Law J W, *et al*. Focused Review: Cytotoxic and Antioxidant potentials of Mangrove-derived *Streptomyces*. *Front Microbiol* 2017, 8: 1-11
- Tornesello A L, Borrelli A, Buonaguro L, *et al*. Antimicrobial Peptides as anticancer agents: Functional properties and biological activities. *Molecules* 2020, 25 (2850): 1-25

- Tyurin A P, Alferova V A, Korshun V A. Chemical elicitors of Antibiotic biosynthesis in Actinomycetes. *Microorganisms* 2018, 6 (2): 1-10
- Vasilchenko A, Julian W, Lapchinskaya O, *et al*. A Novel peptide antibiotic produces by *Streptomyces roseoflavus* strain INA-Ac-5812 with directed activity against gram-positive bacteria. *Front .Microbiol* 2020, 11:1-13
- Verma N P, Dhannidevi, Sinha B, *et al*. Role Microorganisms for the sustainable use of soil pollution abutment in Agriculture lands. *Int. J.Curr. Microbiol. App. Sci* 2017, 6(11): 335-350
- Wahaab F and Subramaniam K. Bioprospecting marine Actinomycetes for multidrug-resistant pathogen control from Rameswaram coastal area, Tamil Nadu, India. *Arch Microbiol* 2017, 2:1-15
- Woo E J, Starks C M, Carney J R, *et al*. Migrastatin and a new compound, isomigrastatin, from *Streptomyces platensis*. *J Antibiot (Tokyo)* 2002, 55(2): 141-6
- Yuf H Q, Li G, Lou H X, *et al*. Isolation, Biosynthesis and Biological activity of polycyclic xanthones from Actinomycetes *Front. Microbiol* 2022, 13:1-35
- Zhang D, Lu Y, Chen H, *et al*. Antifungal peptides produced by actinomycetes and their biological activities against plant diseases. *J Antibiot* 2020, 73, 265–282

Dr. I K Manjula
(Research Guide)

Ms. Akshatha S J
(Research Scholar)